

Escherichia coli K5 heparosan fermentation and improvement by genetic engineering

Zhenyu Wang,¹ Jonathan S. Dordick^{1,3,*} and Robert J. Linhardt^{1,2,4}

Departments of ¹Biology; ²Chemical and Biological Engineering; ³Biomedical Engineering and ⁴Chemistry and Chemical Biology; Center for Biotechnology & Interdisciplinary Studies; Rensselaer Polytechnic Institute; Troy, NY USA

NAcetyl heparosan is the precursor for the biosynthesis of the important anticoagulant drug heparin. The *Escherichia coli* K5 capsular heparosan polysaccharide provides a promising precursor for in vitro chemoenzymatic production of bioengineered heparin. This article explores the improvements of heparosan production for bioengineered heparin by fermentation process engineering and genetic engineering.

Heparosan is an acidic polysaccharide natural product. It is comprised of a [(→4) β-D-glucuronic acid (GlcA) (1→4) *N*-acetyl-α-D-glucosamine (GlcNAc) (1→)]_n repeating disaccharide unit (Fig. 1A). In eukaryotes, heparosan is the precursor polysaccharide in the biosynthesis of heparin and heparan sulfate, biologically important molecules that are involved in blood anticoagulation, viral and bacterial infection and entry, angiogenesis, inflammation, cancer and development.^{1,2}

Heparin (Fig. 1B), a widely used anticoagulant polysaccharide drug biosynthesized through a heparosan intermediate, is currently produced from porcine intestine extract.^{1,3} The animal source production of heparin is associated with several drawbacks, and its risk is evidenced by the heparin contamination crisis in 2008.³ The contamination crisis caused acute side effects in patients, as a result of an adulterant determined to be another similar polysaccharide, oversulfated chondroitin sulfate (OSCS).^{4,5} The in vitro chemoenzymatic synthesis of anticoagulant heparin-like polysaccharide from heparosan has shown promise as an

alternative approach to produce heparin from non-animal source.⁶⁻⁹ Therefore, the production of heparosan is the first step in making bioengineered heparin.¹⁰

Heparosan is biosynthesized as a polysaccharide capsule in bacteria including *Escherichia coli* K5 and *Pasteurella multocida*.¹¹⁻¹³ While the heparosan produced by *P. multocida* is usually of high molecular weight ~200–300 KDa,¹⁴ the *Escherichia coli* K5 heparosan is closer to the size of heparin, with an average molecular weight of 10 KDa to 20 KDa.^{9,10,15} For this reason, we chose *Escherichia coli* K5 as our bacterial strain for further study. The K5 heparosan polysaccharide, comprises the *Escherichia coli* capsule and acts as molecular camouflage, rendering reduced immunogenicity^{11,12} because of its identical structure to mammalian heparosan, the biosynthetic precursor of heparin and heparan sulfate. The initiation of K5 heparosan synthesis reportedly involves 2-keto-3-deoxyoctulosonic acid.¹⁶ K5 heparosan is then elongated through the alternate action of the glycotransferases KfiA and KfiC that add GlcNAc and GlcA to the non-reducing end of the growing polysaccharide chain.^{17,18} The synthesis of heparosan chain may be coupled or at least correlated to the transportation of heparosan to the cell surface.¹⁹⁻²² The heparosan export pathway involves six proteins: KpsC, KpsD, KpsE, KpsM, KpsS and KpsT.²⁰⁻²² The synthesized K5 heparosan chain is believed to be anchored to the cell surface through lipid substitution at the reducing end of the polysaccharide to a phosphatidic acid molecule in the outer membrane of *Escherichia coli*.²³

Key words: heparosan, heparin, K5 capsule polysaccharide, fermentation

Submitted: 10/31/10

Revised: 11/12/10

Accepted: 11/16/10

DOI: 10.4161/bbug.2.1.14201

*Correspondence to: Jonathan S. Dordick;
Email: dordick@rpi.edu

Addendum to: Wang Z, Ly M, Zhang F, Zhong W, Suen A, Hickey AM, et al. *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. *Biotechnol Bioeng* 2010 107:964-73.

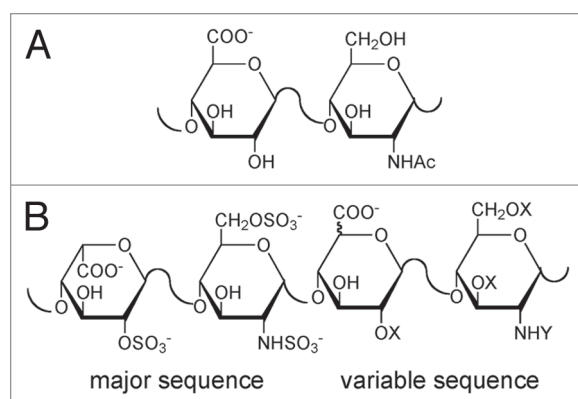


Figure 1. (A) The structure of heparosan disaccharide unit. (B) The structures of the major and minor variable repeating disaccharides comprising heparin where X = SO₃⁻ or H and Y = SO₃⁻ or COCH₃.

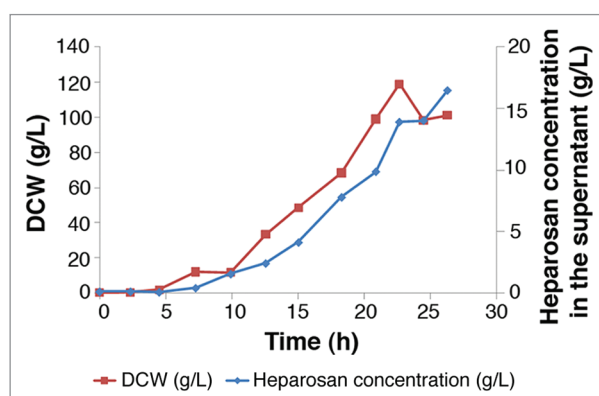


Figure 2. Time course of dry cell weight (g/L) and heparosan concentration in the fermentation supernatant (g/L) during the fermentation in a 20 L fermentor.

Fermentation yield and heparosan chain size are major factors that need to be taken into consideration in order to produce heparosan as a precursor for bioengineered heparin. In our previous study, we have increased the *Escherichia coli* K5 heparosan fermentation yield and productivity, and extensively studied the structure and molecular weight of the heparosan produced.¹⁰ High cell density culturing of *Escherichia coli* using an exponential feeding strategy, can afford up to 15 g/L heparosan at a productivity of 0.4 g/L^h. In traditional exponential feeding, active feedback is absent on the nutritional status of the culture, and there are risks of overfeeding glucose, resulting in the accumulation of toxic byproducts such as acetate, preventing the culture to growing to higher cell density.^{24,25} In a 20 L fermentor with a 15 L working volume, a pH-stat feeding strategy was utilized with culture

pH served as a feedback indicator of the nutritional status of the culture. The culture pH rises when the principal carbon substrate becomes depleted, mainly as a result of the increase in the concentration of ammonium.^{24,26} The feeding pump is turned on only when pH rises above 6.8 to avoid overfeeding. The cell growth and heparosan production courses are presented in Figure 2.

While the K5 heparosan yield and productivity have been significantly increased through fermentation process engineering, it might still be possible to increase heparosan yield and specific productivity through metabolic engineering approaches. The proposed K5 heparosan biosynthetic pathway is presented in Figure 3. Heparosan building blocks GlcA and GlcNAc are used in the form of UDP-GlcA and UDP-GlcNAc, respectively. The UDP sugars are derived

from glucose-6-phosphate. In UDP-GlcA biosynthesis, glucose-6-phosphate is first converted to glucose-1-phosphate, from which cell wall polysaccharide is also synthesized. The reaction of glucose-1-phosphate with UTP yields UDP-glucose. The subsequent conversion of UDP-glucose to UDP-GlcA is catalyzed by the UDP-glucose dehydrogenase, which is encoded by the gene KfiD in the K5 capsule gene cluster.^{27,28} In UDP-GlcNAc biosynthesis glucose-6-phosphate is converted to fructose-6-phosphate. Aminotransferase catalyzes the amino group transfer from glutamine to fructose-6-phosphate. Acetyl group transfer by an acetyltransferase forms *N*-acetylglucosamine-6-phosphate, which is an energy consuming step.²⁹ A mutase then rearranges the phosphate group to form *N*-acetylglucosamine-1-phosphate, and reaction with UTP affords UDP-GlcNAc. The glycotransferase enzymes are encoded by the genes KfiA and KfiC in the K5 capsular gene cluster. These enzymes add GlcNAc and GlcA alternatively to elongate the heparosan chain at the non-reducing end. Two moles of ATP are consumed in two glucokinase reactions to provide a phosphorylated hexose precursor for each branch of the pathway and the other two moles of ATP are utilized to regenerate the donor species UTP. Thus, in this process, a total of 4 mol of ATP are consumed to produce 1 mol of heparosan disaccharide repeating unit. Two moles of NADH are generated from the oxidation reaction catalyzed by UDP-glucose dehydrogenase for each one mole of heparosan disaccharide synthesized. NADH can be oxidized to provide energy. The heparosan biosynthetic pathway shares some common components with the cell wall biosynthetic pathway in *Escherichia coli*, as glucose-1-phosphate is also a precursor in cell wall polysaccharide synthesis and UDP-GlcNAc serves as a peptidoglycan building block.

In the heparosan biosynthesis pathway, the oxidation reaction of UDP-glucose to UDP-GlcA, catalyzed by UDP-glucose dehydrogenase is thought to be the rate limiting step.²⁷ This assumption is based on mammalian glycosaminoglycan biosynthesis, which is regulated by UDP-glucose dehydrogenase activity.²⁷ However, when dehydrogenase was

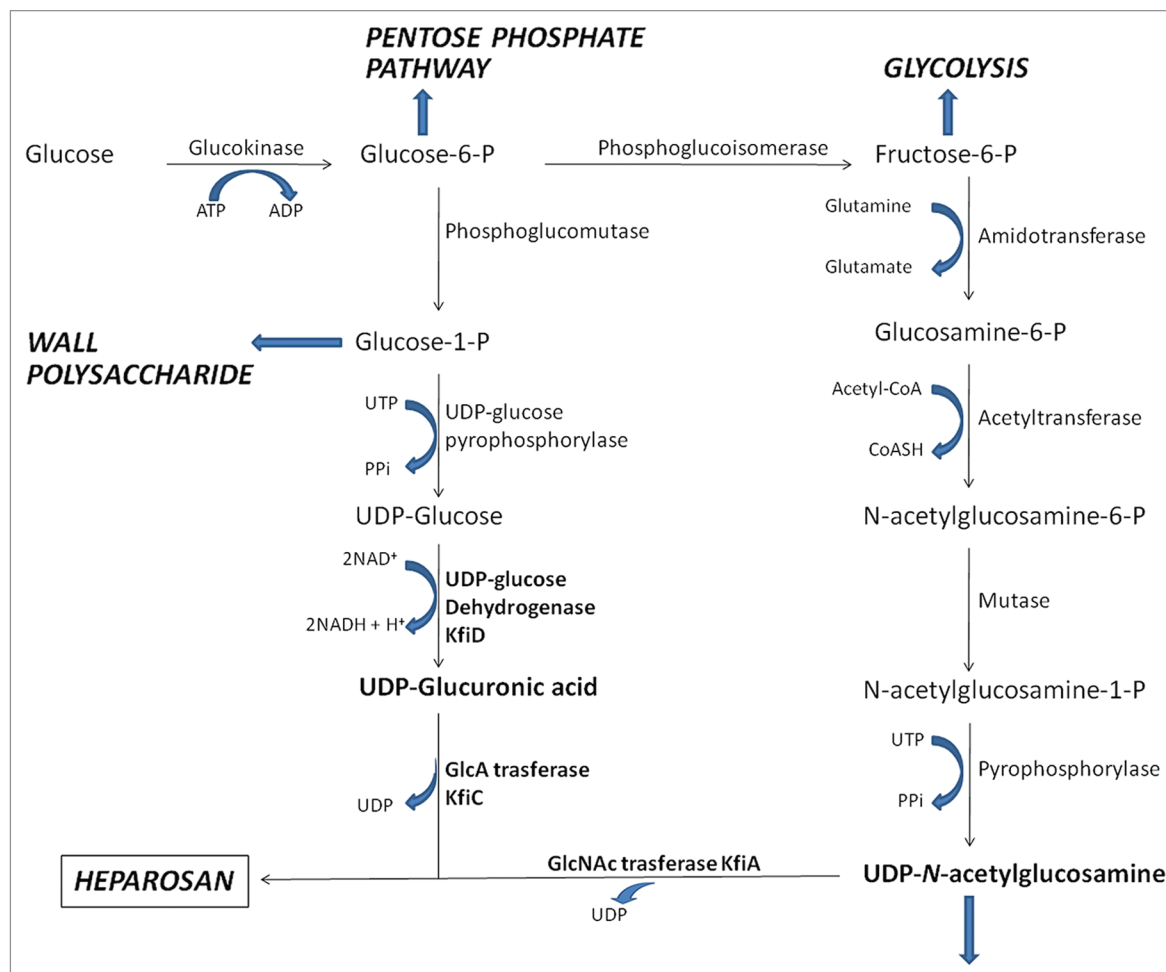


Figure 3. Biosynthesis pathway for heparosan production in *Escherichia coli* K5 strain.

overexpressed in the *Escherichia coli* K5 in an expectation that higher level of UDP-glucose dehydrogenase in the *Escherichia coli* cell would increase the heparosan synthesis, heparosan productivity decreased and the heparosan chain length remained unchanged.²⁷ Overexpression of UDPGDH, while resulting in an increase in UDP-GlcA, may also decrease the pool of glucose 6-phosphate, reducing fructose 6-phosphate, a precursor of UDP-GlcNAc, thus, reducing the rate of K5 biosynthesis initiation, a step that involves the addition of GlcNAc to a membrane acceptor. Another effect of a reduced UDP-GlcNAc pool is a decrease in chain elongation rate, as UDP-GlcNAc becomes rate-limiting for the KfiA transferase. It is also possible that an imbalance in the UDP-GlcNAc and UDP-GlcA concentration may directly interfere with the chain-elongation process. Previous studies

in mammalian heparin biosynthesis have demonstrate that the relative proportions of UDP-GlcNAc and UDP-GlcA impact the rate of heparin polymerization and its chain length.^{27,30}

A successful metabolic engineering strategy needs to balance the two branches of the heparosan biosynthesis pathway, in that an imbalanced supply of UDP-GlcNAc and UDP-GlcA is likely to have a negative effect on the heparosan production. Also, since the heparosan biosynthesis pathway shares common steps with the *Escherichia coli* cell wall biosynthesis, an increase in the mass flow to the production of heparosan will likely to cause the decreased mass flow to cell wall biosynthesis. Bacteria cell wall accounts for a large proportion of the cell dry weight. Thus, it is likely that there is a relatively large reservoir pool of the synthetic pathway components for cell wall synthesis.

Minimally, cells must have the capacity to replenish the supply of the components of cell wall synthesis needed to support rapid cell growth. Thus, cells must have a large pool of UDP-GlcNAc. Assuming the UDP-GlcA pool is balanced with the large supply of UDP-GlcNAc, as required for the efficient synthesis of the heparosan capsule, the UDP-GlcA pool in the cell may also be abundant. In summary, cellular levels of UDP-GlcA and UDP-GlcNAc are probably not limiting in heparosan biosynthesis. It is more likely that the levels and activities of the two glycotransferases, KfiA and KfiC, limit the production of the heparosan. The measurement of intracellular UDP-GlcA and UDP-GlcNAc concentration, the KfiA and KfiC enzymatic activities, and a metabolic flux analysis may be needed to address the validity of this hypothesis. A balanced overexpression of KfiA and KfiC enzymes

may be effective for increasing heparosan production. In this approach, some of the mass flux would be pulled from cell wall synthesis towards heparosan production. The limitation would be a decreased cell growth, as the result of a decreased cell wall synthesis. Thus, an increase in specific productivity of heparosan might be accompanied by reduced cell culture density and lower overall productivity.

Another strategy to increase K5 heparosan specific productivity is to increase the heparosan shedding from the cell surface into the medium. K5 polysaccharide is believed to be retained on the cell surface through lipid substitution at the reducing end of the polysaccharide to a phosphatidic acid molecule in the outer membrane.^{16,23} Heparosan present in the culture supernatant apparently results from either the lability of the phosphodiester linkage or the action of a K5 lyase that breaks down the heparosan chain.^{23,31,32} A mutation on the gene responsible for synthesizing the phosphodiester linkage might increase the concentration of heparosan in the culture supernatant. Unfortunately, the enzyme responsible for the phosphodiester synthesis and the genes encoding the enzyme are unknown. Moreover, it is still not completely clear that heparosan is retained on the cell surface through the phosphodiester bond, as some data suggests the polymer chain may be retained at the surface through ionic and other interactions.³³ A transposon mutagenesis study, demonstrated that a mutation in the *WaaR* gene had an effect on the cell surface retention of K5 heparosan, increasing its content in the culture supernatant from 33–77%.³⁴ The *WaaR* protein is related to the lipopolysaccharide outer core biosynthesis, and its impact on heparosan retention suggests that interactions between the outer core and the heparosan capsule are important in maintaining the overall cell surface architecture.³⁴ A mutant strain could be valuable to the commercial production of heparosan.

The K5 lyase might also be useful to enhance the heparosan release from the cell surface. K5 lyase is produced by *Escherichia coli* bacterial phage, and cleaves heparosan chain through a β -elimination mechanism.³¹ The lyase gene is integrated into the *Escherichia coli* K5 DNA.¹⁵ An

overexpression of the lyase might increase the heparosan concentration in the culture supernatant by cutting heparosan chains from the cell surface. However, the action of lyase can adversely impact the molecular weight of heparosan and introduces a double bond into the non-reducing end of the heparosan chain. If the goal of heparosan production is to prepare a precursor for bioengineered generic heparin, an alteration in polysaccharide molecular weight and the introduction of unnatural unsaturated saccharide residue are an unwelcome result of this approach.

Heparin chain size plays a critical role in mediating its binding to proteins in blood anticoagulation. Thus, the chain size of the heparosan precursor is a critical property. Understanding how the heparosan chain is terminated in biosynthesis would be helpful in developing methods to control heparosan chain size. Unfortunately, there are limited data on the chain termination mechanism, and it is not clear how the heparosan chain length is controlled by *Escherichia coli*. The ratio of UDP-GlcA and UDP-GlcNAc and chain polymerization rate appear to be dominant factors in the control of chain length in mammalian heparin biosynthesis, suggesting that the same factors might play a role in *Escherichia coli*.³⁰

Instead of using biosynthesis to control heparosan chain length, it might be possible to exert catabolic control using K5 lyase. Previous studies have shown that the activity of K5 lyase expressed by the *Escherichia coli* was affected by growth media and culture conditions.^{10,15,32} However, the mechanism to control the lyase expression is not clear. It seems the lyase is present throughout the entire time course of the fermentation. An improved *Escherichia coli* strain with controllable K5 lyase expression might result in heparosan of more defined chain size. The first step would be to delete the K5 lyase gene, which is integrated into the K5 *Escherichia coli* genome, and to construct an inducer controlled expression vector. Previous studies have shown that the K5 lyase exist in both an intracellular form and an extracellular form.^{15,32} This suggests that *Escherichia coli* has the machinery to export the lyase to the extracellular space. A new K5 expression system would be needed that would

first allow the *Escherichia coli* cells to grow to certain cell density required for the production of large amount of heparosan. Subsequent addition of an inducer would result in the expression of K5 lyase and the culture would enter a phase for chain size modulation, which could then be terminated through the deactivation of K5 lyase once the desired heparosan chain size was obtained.

The second problem with the use of K5 lyase for heparosan molecular weight control is the undesirable introduction of a double bond in the non-reducing end of the heparosan chain. This double bond needs to be removed to prepare a bioengineered heparin. While this double bond can be eliminated from heparosan by deleting the gene of K5 lyase from the K5 *Escherichia coli* genome, this would result in a decrease of heparosan in the culture supernatant and an increase in heparosan chain size. While it is possible to control chain size and remove the unsaturated sugar from heparosan by chemical methods using hydrogen peroxide or ozone,³⁶ metabolic engineering offers an alternative solution. The double bond can also be removed with $\Delta 4,5$ -glycuronidase, an unusual enzyme that hydrolyzes the unsaturated $\Delta 4,5$ uronic acid at the nonreducing end of polysaccharides.³⁷ This gene, from *Flavobacterium heparinum*, has been recombinantly expressed in *Escherichia coli*.³⁷ This enzyme could be used to directly treat heparosan to remove its unsaturated non-reducing end. Alternatively, the gene for this enzyme could be introduced into the same controllable lyase expression system mentioned above. So that as the K5 lyase acts to release heparosan into the culture medium, the $\Delta 4,5$ -glycuronidase removes the unsaturated sugar unit from the released heparosan. This would need an expression system that can secrete the active enzyme into extracellular space so to gain contact with the extracellular heparosan.^{38,39} Moreover, the induction of lyase expression should use an *Escherichia coli* metabolizable inducer to terminate the lyase expression before stopping $\Delta 4,5$ -glycuronidase expression, leaving enough time for $\Delta 4,5$ -glycuronidase to remove all unsaturated sugar unit from the heparosan.

In conclusion, a combination of fermentation process engineering and strain

improvement by genetic engineering and metabolic engineering may represent an excellent strategy to afford heparosan with ideal structural properties and in excellent yields for use as a precursor for bioengineered heparin production.

Acknowledgements

This work was funded in part by the National Institutes of Health (HL096972).

References

- Linhardt RJ, Claude S. Hudson Award address in carbohydrate chemistry. Heparin: Structure and activity. *J Med Chem* 2003; 46:2551-64.
- Linhardt RJ, Toida T. Role of glycosaminoglycans in cellular communication. *Acc Chem Res* 2004; 37:431-8.
- Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. *Nat Prod Rep* 2009; 26:313-21.
- Kishimoto TK, Viswanathan K, Ganguly T, Elankumaran S, Smith S, Pelzer K, et al. Contaminated heparin associated with adverse clinical events and activation of the contact system. *New Engl J Med* 2008; 358:2457-67.
- Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, et al. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nat Biotechnol* 2008; 26:669-75.
- Lindahl U, Li JP, Kusche-Gullberg M, Salmivirta M, Alaranta S, Veromaa T, et al. Generation of "Neoheparin" from *E. coli* K5 capsular polysaccharide. *J Med Chem* 2005; 48:349-52.
- Kuberan B, Beeler DL, Lech M, Wu ZL, Rosenberg RD. Chemoenzymatic synthesis of classical and non-classical anticoagulant heparan sulfate polysaccharides. *J Biol Chem* 2003; 278:52613-21.
- Chen J, Avci FY, Munoz EM, McDowell LM, Chen M, Pedersen LC, et al. Enzymatic redesigning of biologically active heparan sulfate. *J Biol Chem* 2005; 280:42817-25.
- Zhang ZQ, McCallum SA, Xie J, Nieto L, Corzana F, Jimenez-Barbero J, et al. Solution structures of chemoenzymatically synthesized heparin and its precursors. *J Am Chem Soc* 2008; 130:12998-3007.
- Wang Z, Ly M, Zhang F, Zhong W, Suen A, Hickey AM, et al. *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. *Biotechnol Bioeng* 2010; 107:968-77.
- Vann WF, Schmidt MA, Jann B, Jann K. The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* 010:K5:H4. A polymer similar to desulfo-heparin. *Eur J Biochem* 1981; 116:359-64.
- Manzoni M, Bergomi S, Cavazzoni V. Extracellular K5 Polysaccharide of *Escherichia Coli*—Production and Characterization. *J Bioact Compat Polym* 1993; 8:251-7.
- DeAngelis PL, White CL. Identification and molecular cloning of a heparosan synthase from *Pasteurella multocida* type D. *J Biol Chem* 2002; 277:7209-13.
- Deangelis PL. Heparosan-based biomaterials and coatings and methods of production and use. *PCT Int Appl* 2009; 2009014559.
- Manzoni M, Bergomi S, Cavazzoni V. Production of K5 polysaccharides of different molecular weight by *Escherichia coli*. *J Bioact Compat Polym* 1996; 11:301-11.
- Finke A, Bronner D, Nikolaev AV, Jann B, Jann K. Biosynthesis of the *Escherichia coli* K5 polysaccharide, a representative of group II capsular polysaccharides: Polymerization in vitro and characterization of the product. *J Bacteriol* 1991; 173:4088-94.
- Sugiura N, Baba Y, Kawaguchi Y, Iwatani T, Suzuki K, Kusakabe T, et al. Glucuronyltransferase activity of KfiC from *Escherichia coli* strain K5 requires association of KfiA: KfiC and KfiA are essential enzymes for production of K5 polysaccharide, *N*-acetylheparosan. *J Biol Chem* 2010; 285:1597-606.
- Hodson N, Griffiths G, Cook N, Pourhossein M, Gottfridson E, Lind T, et al. Identification that KfiA, a protein essential for the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide, is an alpha-UDP-GlcNAc glycosyltransferase. The formation of a membrane-associated K5 biosynthetic complex requires KfiA, KfiB and KfiC. *J Biol Chem* 2000; 275:27311-5.
- Whitfield C, Roberts IS. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol* 1999; 31:1307-19.
- Arrecubieta C, Hammarton TC, Barrett B, Chareonsudjai S, Hodson N, Rainey D, et al. The transport of group 2 capsular polysaccharides across the periplasmic space in *Escherichia coli*. Roles for the KpsE and KpsD proteins. *J Biol Chem* 2001; 276:4245-50.
- McNulty C, Thompson J, Barrett B, Lord L, Andersen C, Roberts IS. The cell surface expression of group 2 capsular polysaccharides in *Escherichia coli*: The role of KpsD, RhsA and a multi-protein complex at the pole of the cell. *Mol Microbiol* 2006; 59:907-22.
- Silver RP, Prior K, Nshahai C, Wright LF. ABC transporters and the export of capsular polysaccharides from gram-negative bacteria. *Res Microbiol* 2001; 152:357-64.
- Jann B, Jann K. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. *Curr Top Microbiol Immunol* 1990; 150:19-42.
- Lee SY. High cell-density culture of *Escherichia coli*. *Trends Biotechnol* 1996; 14:98-105.
- Johnston WA, Stewart M, Lee P, Cooney MJ. Tracking the acetate threshold using DO-transient control during medium and high cell density cultivation of recombinant *Escherichia coli* in complex media. *Biotechnol Bioeng* 2003; 84:314-23.
- Lee SY, Chang HN. High cell density cultivation of *Escherichia coli* W using sucrose as a carbon source. *Biotechnol Lett* 1993; 15:971-4.
- Roman E, Roberts I, Lidholt K, Kusche-Gullberg M. Overexpression of UDP-glucose dehydrogenase in *Escherichia coli* results in decreased biosynthesis of K5 polysaccharide. *Biochem J* 2003; 374:767-72.
- Schiller JG, Lamy F, Frazier R, Feingold DS. UDP-glucose dehydrogenase from *Escherichia coli*. Purification and subunit structure. *Biochim Biophys Acta* 1976; 453:418-25.
- Chong BF, Blank LM, McLaughlin R, Nielsen LK. Microbial hyaluronic acid production. *Appl Microbiol Biotechnol* 2005; 66:341-51.
- Lidholt K, Riesenfeld J, Jacobsson KG, Feingold DS, Lindahl U. Biosynthesis of heparin. Modulation of polysaccharide chain length in a cell-free system. *Biochem J* 1988; 254:571-8.
- Hanfling P, Shashkov AS, Jann B, Jann K. Analysis of the enzymatic cleavage (beta elimination) of the capsular K5 polysaccharide of *Escherichia coli* by the KS-specific coliphage: A reexamination. *J Bacteriol* 1996; 178:4747-50.
- Manzoni M, Bergomi S, Rollini M. Influence of the culture conditions on extracellular lyase activity related to K5 polysaccharide. *Biotechnol Lett* 2000; 22:81-5.
- Whitfield C. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu Rev Biochem* 2006; 75:39-68.
- Taylor CM, Goldrick M, Lord L, Roberts IS. Mutations in the waaR gene of *Escherichia coli* which disrupt lipopolysaccharide outer core biosynthesis affect cell surface retention of group 2 capsular polysaccharides. *J Bacteriol* 2006; 188:1165-8.
- Ly M, Wang Z, Laremore TN, Zhang F, Zhong W, Pu D, et al. Analysis of *E. coli* K5 capsular polysaccharide heparosan. *Anal Bioanal Chem* 2010; 399:737-45.
- Rota C, Liverani L, Spelta F, Mascellani G, Tomasi A, Iannone A, et al. Free radical generation during chemical depolymerization of heparin. *Anal Biochem* 2005; 344:193-203.
- Myette JR, Shriver Z, Kiziltepe T, McLean MW, Venkataraman G, Sasisekharan R. Molecular cloning of the heparin/heparan sulfate delta 4,5-unsaturated glucuronidase from *Flavobacterium heparinum*, its recombinant expression in *Escherichia coli*, and biochemical determination of its unique substrate specificity. *Biochemistry* 2002; 41:7424-34.
- Lee CC, Wong DW, Robertson GH. An *E. coli* expression system for the extracellular secretion of barley alpha-amylase. *J Protein Chem* 2001; 20:233-7.
- Khushoo A, Pal Y, Singh BN, Mukherjee KJ. Extracellular expression and single step purification of recombinant *Escherichia coli* L-asparaginase II. *Protein Expr Purif* 2004; 38:29-36.